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13. ABSTRACT (Maximum 200 Words)
Studies on the structure of acetylcholinesterase (AChE) as a target for organophosphate toxicity continue and have resulted in several leads of significance. First, we have completed the first phase of studies of the structural determinants on the enzyme responsible for inactivation and oxime reactivation. The oxime reactivation studies reveal mutations whereby reactivation can be accelerated some 100-fold, and the combination oxime-mutant AChE shows promise as a prophylactic agent or antidote. Second, through cysteine-substitution mutagenesis and labeling, we have developed steady-state and anisotrophy decay fluorescence methods to examine segmental motion in the protein. This has yielded valuable information on the flexibility of the active center gorge, and the practical outcome of a potentially high sensitivity detection method for organophosphate exposure using the enzyme target as a detector. Third, we have developed an analytical means of measuring organophosphate exposure by MALDI-TOF mass spectrometry that actually measures the product rather than drawing inferences from inhibition of activity. Lastly, several other studies related to our overall objectives are underway. The first is the production of gene knockouts for all of the cholinesterase splice variants.

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Acetylcholinesterase, organophosphate, oxime reactivation, fluorescence spectroscopy, nerve agent antidotes, exposure detection

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INTRODUCTION

The global objective of this project is to understand the structure of acetylcholinesterase in solution so that both inhibition by nerve agent organophosphates and reactivation by oximes and other antidote nucleophiles can be understood at a molecular level. Moreover, the chemical and physical procedures used may lead to improved detection of organophosphate exposure from remote sites. The approach is based on the premise that, while crystal structures provide a template of the organization of the atoms under the constraints of crystallization,fluctuations in solution yield structures that diverge substantially from the crystal structure, and these structural fluctuations are critical to understanding selectivity of catalysis and inhibition.

BODY

Considerable progress has been made on several fronts. The project areas will be considered individually.

1. Analysis of Dynamics of Acetylcholinesterase in Relation to Catalysis and Inhibition.

The location of the active center of acetylcholinesterase (AChE) at the base of a 20 Å, narrow gorge in relation to diffusion limited rates of inhibitor entry and high efficiency catalysis has presented a long-standing enigma. Alternate portals of entry or exit for substrate and product have been proposed, but lack either a thermodynamic foundation or experimental evidence. In fact, the mutagenesis evidence points to a single portal of entry for all substrates. These observations lead to the consideration that either the crystallographic structures preferentially produce a closed gorge conformation or extensive breathing motions in the molecule give rise to transient gorge openings enabling the ligand to rapidly pass. Resolving this question will require physical methods that go beyond simple mutagenesis and measurement of catalytic parameters.

To this end, we have developed a physical approach to measure the effective dielectric constant of substituted side chains through fluorescence spectroscopy (Boyd et al., 2000; Shi et al., 2001, 2002). The studies are based on cysteine substitution mutagenesis of selected residues in the molecule. Since the other cysteines in mammalian AChE are locked in disulfide bonds, the introduced cysteine is labeled with an appropriate fluorophore. We have used acrylodan primarily for this purpose since its excited state dipole is oriented in such a way that it becomes highly sensitive to the dielectric constant of the medium.

This approach has been complemented recently by measurements of side chain dynamics through the decay of fluorescence anisotropy in the nanosecond time frame. AChE is a member of the α,β hydrolase fold family. Within this family, an omega loop that outlines the active center gorge shows conformational changes upon substrate binding and distinctive structural characteristics among the hydrolases in the family. Initially, we have concentrated on this region. Studies involving the analysis of the

effective dielectric constants in the microenvironment around the substituted fluorescent side chain have been detailed in a previous progress report (Boyd et al., 2000), but point to a conformational change in which the active center gorge envelops around the bound ligand (Shi et al., 2001). In the case of the omega loop, the fluorescence studies show that the inner portion excludes solvent water upon ligand binding, as would be predicted from the ligand occupation and the closure of the gorge around the ligand. More importantly, the outer portion of the omega loop exhibits increased curvature causing the side chains to move into the solvent, thereby showing a bathochromic emission shift of the acrylodan residue (Shi et al., 2001, 2002). The residues showing this change in environment are a considerable distance from the binding site, so the conformational change is truly allosteric in character. Also, examination of three residues from the tip of the loop to the outer side of its base (76, 81 and 84) indicates a degree of plasticity in the fluctuations of the loop rather than it behaving as a rigid flap.

Residues moving from a hydrophobic surface into the bulk solvent would be expected to show enhanced segmental and torsional motions, whereas those residues at the ligand binding interface upon binding of ligand would become more immobilized. Indeed, this is found to be the case in our measurements of anisotropy decay using fluorescein and anilinosulfonates as probes conjugated to specified cysteines on AChE. The intrinsic fluctuations differ through the substituted positions in the molecule, where those in the internal wall of the gorge show less segmental and torsional motion, whereas those on outer surfaces and loop tips show greater motion (Shi et al., 2003; Boyd et al., in preparation). Binding of ligand shows immobilization of those side chains at the ligand-macromolecule interface (Shi et al., 2003), whereas those on the outer portion of the omega loop show an enhanced rate of decay of anisotropy and increased segmental motion. The fluorescence lifetimes, anisotropy decay times and amplitudes of the phases of decay have been analyzed and presented in great detail (Shi et al., 2003). The data are consistent with structure and correlate reasonably well with molecular dynamics analysis of the vectoral movements of the side chains.

2. Development of a Sensitive Fluorescence Detection System for Organophosphate Conjugation

Since reversible ligands cause characteristic changes in the spectra of the individual residues, we also analyzed the spectral characteristics of acrylodan in organophosphate conjugated AChE. Here, we observed distinctive changes in acrylodan emission spectra at the 81 position that can distinguish the nature of the conjugating ligand. Moreover, enantiomeric selectivity can be discriminated (Shi et al., 2002). The molecular explanation for this stems from acyl pocket dimensions and whether the conjugated ligand can fit in the pocket and simultaneously have the phosphoryl or carbonyl oxygen enter the oxyanion hole. Since the cut off for acyl esters fitting in the acyl pocket and being efficiently hydrolyzed by AChE is between the propionyl and butyryl groups, the methoxyphosphoryl, but not ethoxyphosphoryl moiety will associate within the acyl pocket. Similarily the Sp-methylphosphonyl esters, but not the corresponding Rp-enantiomers, will fit in the binding site occupying the acyl pocket and oxyanion hole. Those compounds achieving the fit produce a bathochromic (red) shift in emission

spectra, while those unable to accommodate a fit produce a hypsochromic (blue) shift. The shifts are of sufficient magnitude that the respective conjugates can serve as a sensitive detector of volatile organophosphates (Shi et al., 2003).

Hence, we have developed a potential remote sensor of organophosphate exposure that cannot only detect conjugation, but can distinguish certain conjugates. The sensor is the very target of organophosphate toxicity.

3. The role of active center and peripheral site determinants in the reactivity of organophosphates, spontaneous reactivation, aging and oxime-mediated reactivation of the conjugates.

This has been an ongoing study that has expanded to include multiple mutations in order to develop an optimal mutant AChE-oxime combination that will serve in a scavenging capacity for organophosphate toxicity. The study includes several combinatorial mutants of the acyl pocket (F295, F297), choline binding subsite (Y337, Y338) and the negative site proximal to the active center serine (E202). The study of inactivation has been published (Kovarik et al., 2003a) where we showed these mutations have a far greater effect on the binding parameters for the enantiomeric organophosphate than substrate. Certain combinations of mutants show greater alterations in organophosphate binding parameters than substrate catalysis. Stereoselectivity is typically diminished upon mutation at particular positions. A decrease in Sp reactivity is seen, whereas Rp reactivity is enhanced. In some cases (F297I, F297I combined with Y337A) the stereochemical preference is inverted where the Rp enantiomer becomes the more reactive compound.

The reactivation study, which has been more extensively developed and analyzed during this project period (Kovarik et al., 2003b) has also established that enhancing selective dimensions of the active center gorge through combinatorial mutations increases reactivation rates substantially, while excessive clearance in the gorge, such as that in found in butyrylcholinesterase, results in a decrease in reactivation rate.

In general, stereospecificity in reactivation is altered with the mutation, but the more reactive Sp enantiomer still forms the conjugate that is most amenable to reactivation. Hence, activation and reactivation pass through a similarly oriented transition state. With the wild type enzyme, the bimolecular rate constants for reactivation vary from 10 $M^{-1}min^{-1}$ to 50 x $10^4 M^{-1}min^{-1}$ for HI-6, generally the most effective oxime nucleophile. Similar rates of reactivation by 2-PAM and HI-6 are observed for the Rp-isopropyl methyl conjugates ($1.5M^{-1}min^{-1}$). The smaller alkyl moieties for the three Sp enantiomers show the most rapid rates of activation, presumably because of minimal steric interference for the positioning of the attacking nucleophile in the oxime. The mutation in the choline binding site (Y337A) accelerated reactivity of both oximes for virtually all of the Sp and Rp conjugates. In fact, this mutation conferred reactivatibility to certain Rp conjugates that resist reactivation. The fractional increase in reactivation upon mutation was greatest for the more bulky conjugates, and maximally a 120-fold enhancement in reactivation rate can be achieved through the Y337A, Y297I double mutation for the

cycloheptylmethylphosphono-AChE conjugate. The rate constants so achieved (Tables 1 – 6), bring us in a range where the combination of the mutated AChE and the HI-6 oxime combination can be tried in various scavenging stratagems both *in- vivo* and *in-vitro* for organophosphate exposure and toxicity. Reactivation has been analyzed with respect to oxime structure and mutation, and in several cases, it has been possible to deconstruct the overall bimolecular rate constant into an equilibrium constant for association K_{ox} and an instrinsic unimolecular constant for the bond making or breaking process k_2 (Kovarik et al., 2003a & b).

As an adjunct to these studies, we have developed a matrix associated laser desorption ionization (MALDI) mass spectrometric method for detection of the organophosphate toxicity. One of the major hindrances to measuring organophosphate exposure stems from the variability in AChE activity in biological samples, hence precluding the measurement of low levels of exposure. Fractional reductions in activity are obscured by the variation in AChE activity in the various animal species. Moreover, it has been our experience that this problem prevails even in individual animal strains. By contrast, measurement of the conjugate levels starts from a zero endpoint, where one detects the accumulation of the inhibited enzyme directly, in the form of the covalent conjugate that the organophosphate forms.

The mass spectrometric method has provided a direct measure of inactivation, spontaneous reactivation and aging. To date, we have studied DDVP, paraoxon and DFP that form the dimethoxy, diethoxy and diisopropoxy phosphoryl-conjugates, respectively (Jennings et al., 2003). From this approach we are able to detect rates of: (a) formation of the initial dialkoxy conjugate, (b) aging with the concomitant formation of the monoalkoxy conjugate, and (c) reactivation with the formation of the parent enzyme with the reactive serine at its active site. Surprisingly, we note substantial aging of the dimethoxy conjugate. The MALDI technique relies on digestion for formation of the tryptic, S203 containing, active center peptide at 4321 Da, and the addition of the respective molecular weights to the peptide from the three conjugates and the subsequent partial loss of mass mediated by aging.

Moreover, we have demonstrated that such measurements contain the inherent sensitivity that they can be made on intact tissues with amounts as small as a single mouse brain. Purification has involved affinity chromatography and antibody selection depending on the particular conjugate. The procedure also requires the purification of the tryptic peptide by reverse phase chromatography. Using this procedure, one can get proportionate recovery of all of the tryptic peptides with equivalent vaporization. This has enabled us to expose mice to organophosphate at multiple doses, sacrifice the animal, extract and purify the AChE from total brain or a brain hemisphere and ascertain the amount of free enzyme, aged and non-aged conjugates in the tissue following exposure. The procedure pushes the limit of detection, since AChE makes up 1/30,000 of the mole fraction of proteins in the mouse brain proteome. The AChE knockout animal without organophosphate exposure has been used as the appropriate control in these studies.

4. Transgenic Animal Studies

As an adjunct to these studies we are developing four transgenic animal strains for general use by the research community. All are deletion mutants, where exon 5, exon 6 and exons 5 and 6 have been deleted to eliminate the options for splicing. Although all of the phenotyping data are not complete, we have been able to observe that the animals are more robust than the total AChE knock out (Lockridge et al. 2000) The transgenic animals have been made as conditional knockouts, which will enable us in some cases to obtain a tissue specific knockout. It is also evident that small quantities of AChE expressed in the tissue greatly improve the condition of the animals, suggesting that large changes in levels of AChE can be tolerated under homeostatic conditions. These animals should prove to be extremely valuable in delineating the mechanism of and locus of toxicity in central and peripheral systems following organophosphate exposure.

Table 1: Reactivation of recombinant DNA-derived mouse cholinesterases phosphonylated with S_P-cycloheptylmethyl phosphonyl thiocholine[a]

Reactivator [mM]	Enzyme	k_{+2} / min^{-1}	K_{ox} / mM	k_r / $min^{-1}M^{-1}$	React.$_{max}$ (Time) / %	
HI-6 [0.2-20]	AChE w.t.	0.60±0.04	5.4±0.8	112±19	90	(5 min)
HI-6 [0.02-0.5]	Y337A	-	-	2000±90	80	(1 min)
HI-6 [0.002-0.1]	F295L/Y337A	-	-	13180±1414	80	(3 min)
HI-6 [0.01-5]	F297I/Y337A	6.0±0.5	2.6±0.4	2300±400	100	(1 min)
HI-6 [0.2-10]	Y337A/F338A	0.051±0.003	0.50±0.12	102±26	80	(30 min)
HI-6 [10]	F295L/F297I/Y337A[b]	-	-	-	80	(20 min)
HI-6 [1, 30]	BChE w.t.	-	-	-	<10	(48 h)
2-PAM [1-20]	AChE w.t.	0.0040±0.0007	6.1±3.0	0.66±0.34	70	(15 h)
2-PAM [0.1-40]	Y337A	0.0025±0.0001	0.62±0.14	4.1±1.0	80	(5 h)
2-PAM [0.4-20]	F295L/Y337A	0.016±0.003	10±3	1.7±0.5	70	(3 h)
2-PAM [2-30]	F297I/Y337A	0.018±0.002	2.7±1.2	6.9±3.3	70	(2 h)
2-PAM [1-60]	Y337A/F338A	0.00035±0.00002	0.75±0.45	0.47±0.29	40	(25 h)
2-PAM [40]	F295L/F297I/Y337A[b]	-	-	-	100	(5 h)
2-PAM [5,40]	BChE w.t.	-	-	-	<5	(48 h)

[a] Constants (± standard errors) are calculated (Eqns. (1)-(3)) from k_{obs} constants (8-16 values) obtained in 2-7 experiments. [b] Only one k_{obs} determined.

Table 2: Reactivation of recombinant DNA-derived mouse cholinesterases phosphonylated with S_P-isopropyl methyl phosphonyl thiocholine[a]

Reactivator [mM]	Enzyme	k_{+2} / min^{-1}	K_{ox} / mM	k_r / $min^{-1}M^{-1}$	$React._{max}$ / %	(Time)
HI-6 [0.05-1]	AChE w.t.	0.20±0.03	0.15±0.09	1330±780	90	(10 min)
HI-6 [0.2-20]	Y337A	1.13±0.08	4.7±0.8	240±47	100	(2 min)
HI-6 [0.5-30]	F295L/Y337A	0.27±0.01	0.37±0.09	730±180	80	(10 min)
HI-6 [0.05-1]	F297I/Y337A	0.95±0.13	0.41±0.14	2330±844	100	(5 min)
HI-6 [1-20]	Y337A/F338A	0.26±0.02	1.5±0.6	178±74	90	(15 min)
HI-6 [0.05-5]	BChE w.t.	0.014±0.001	0.064±0.024	215±70	80	(90 min)
2-PAM [0.1-10]	AChE w.t.	0.095±0.013	0.088±0.075	1080±940	100	(30 min)
2-PAM [0.2-40]	Y337A	0.21±0.01	2.6±0.3	82±11	80	(20 min)
2-PAM [1-40]	F295L/Y337A	-	-	3.5±0.4	90	(30 min)
2-PAM [0.1-40]	F297I/Y337A	2.9±0.2	5.5±1.3	534±133	90	(1 min)
2-PAM [1-40]	Y337A/F338A	0.072±0.004	1.5±0.5	46±14	90	(30 min)
2-PAM [0.05-10]	BChE w.t.	2.98±0.01	2.39±0.02	1250±9	90	(1 min)

[a] Constants (± standard errors) are calculated (Eqns. (1)-(3)) from k_{obs} constants (6-19 values) obtained in 2-5 experiments.

Table 3: Reactivation of recombinant DNA-derived mouse cholinesterases phosphonylated with S_P-3,3-dimethylbutyl methyl phosphonyl thiocholine[a]

Reactivator [mM]	Enzyme	k_{+2} min^{-1}	K_{ox} mM	k_r $min^{-1}M^{-1}$	React.$_{max}$ %	(Time)
HI-6 [0.05-5]	AChE w.t.	0.39±0.09	3.8±1.8	102±53	80	(10 min)
HI-6 [0.05-5]	Y337A	3.2±0.5	2.8±1.0	1200±490	100	(2 min)
HI-6 [0.01-1]	F295L/Y337A	-	-	1300±60	100	(2 min)
HI-6 [0.01-10]	F297I/Y337A	1.4±0.1	1.9±0.5	720±180	100	(5 min)
HI-6 [1-40]	Y337A/F338A	0.10±0.01	2.1±1.2	47±28	80	(20 min)
HI-6 [10]	F295L/F297I/Y337A[b]	-	-	-	80	(10 min)
HI-6 [0.5-20]	BChE w.t.	0.73±0.14	7.1±3.2	103±51	100	(10 min)
2-PAM [1-40]	AChE w.t.	-	-	0.18±0.01	90	(8 h)
2-PAM [5-40]	Y337A	-	-	0.041±0.003	80	(33 h)
2-PAM [0.5-40]	F295L/Y337A	0.0018±0.0002	2.8±1.1	0.66±0.27	90	(25 h)
2-PAM [10-60]	F297I/Y337A	0.0025±0.0002	9.0±3.6	0.27±0.11	80	(15 h)
2-PAM [5-60]	Y337A/F338A	0.00023±0.00001	0.54±0.85	0.42±0.65	60	(70 h)
2-PAM [40]	F295L/F297I/Y337A[b]	-	-	-	90	(6 h)
2-PAM [3-30]	BChE w.t.	0.028±0.003	3.2±1.7	8.7±4.6	100	(3 h)

[a] Constants (± standard errors) are calculated (Eqns. (1)-(3)) from k_{obs} constants (5-15 values) obtained in 2-4 experiments. [b] Only one k_{obs} determined.

Table 4: Reactivation of recombinant DNA-derived mouse cholinesterases phosphonylated with R_P-cycloheptyl methyl phosphonyl thiocholine[a]

Reactivator [mM]	Enzyme	k_{+2} / min^{-1}	K_{ox} / mM	k_r / $min^{-1}M^{-1}$	React.$_{max}$ / %	(Time)
HI-6 [1, 40]	AChE w.t.	-	-	-	<15	(50 h)
HI-6 [0.3-20]	Y337A	0.00042±0.00002	1.0±0.2	0.41±0.06	50	(85 h)
HI-6 [1-30]	F295L/Y337A	-	-	-	<25	(40 h)
HI-6 [10-40]	F297I/Y337A	-	-	-	<25	(72 h)
HI-6 [0.2-2]	Y337A/F338A	-	-	-	<15	(40 h)
HI-6 [10-30]	BChE w.t.	-	-	-	<15	(50 h)
2-PAM [1, 40]	AChE w.t.	-	-	-	<25	(50 h)
2-PAM [0.3-5]	Y337A	0.00047±0.00004	0.36±0.16	1.3±0.6	50	(85 h)
2-PAM [20-40]	F295L/Y337A	-	-	-	<25	(40 h)
2-PAM [5-40]	F297I/Y337A	-	-	-	<40	(60 h)
2-PAM [0.3-5]	Y337A/F338A	0.00040±0.00010	0.99±0.87	0.40±0.37	40	(20 h)
2-PAM [20-40]	BChE w.t.	-	-	0.027±0.001	70	(25 h)

[a] Constants (± standard errors) are calculated (Eqns. (1)-(3)) from k_{obs} constants (4-8 values) obtained in 1-3 experiments.

Table 5: Reactivation of recombinant DNA-derived mouse cholinesterases phosphonylated with R_P-isopropylmethyl phosphonyl thiocholine[a]

Reactivator [mM]	Enzyme	k_{+2} / min^{-1}	K_{ox} / mM	k_r / $min^{-1}M^{-1}$	React.$_{max}$ / %	(Time)
HI-6 [0.2-40]	AChE w.t.	0.0075±0.0003	4.3±0.7	1.7±0.3	70	(10 h)
HI-6 [0.2-30]	Y337A	0.0071±0.0004	0.97±0.23	7.3±1.8	80	(8 h)
HI-6 [1-30]	F295L/Y337A	0.0013±0.0001	0.95±0.56	1.3±0.8	70	(16 h)
HI-6 [5-40]	F297I/Y337A	0.0021±0.0003	16±8	0.13±0.07	50	(16 h)
HI-6 [1-30]	BChE w.t.	0.00035±0.00004	1.1±0.9	0.23±0.25	40	(25 h)
2-PAM [0.3-40]	AChE w.t.	0.0029±0.0004	1.9±1.1	1.5±0.9	70	(20 h)
2-PAM [0.3-40]	Y337A	0.0026±0.0001	0.60±0.21	4.3±1.5	80	(8 h)
2-PAM [5-30]	F295L/Y337A	0.0096±0.0039	10±11	0.95±1.09	80	(10 h)
2-PAM [5-60]	F297I/Y337A	-	-	0.041±0.003	100	(25 h)
2-PAM [5-30]	BChE w.t.	0.024±0.006	27±13	0.88±0.47	100	(4 h)

[a] Constants (± standard errors) are calculated (Eqns. (1)-(3)) from k_{obs} constants (6-10 values) obtained in 2-4 experiments.

Table 6: Reactivation of recombinant DNA-derived mouse cholinesterases phosphonylated with R_P-3,3-dimethylbutylmethyl phosphonyl thiocholine[a]

Reactivator [mM]	Enzyme	k_{+2} / min^{-1}	K_{ox} / mM	k_r / $min^{-1}M^{-1}$	React.$_{max}$ / %	(Time)
HI-6 [0.2-2]	AChE w.t.	-	-	-	<15	(40 h)
HI-6 [0.2-40]	Y337A	0.00040±0.00002	0.54±0.14	0.74±0.19	70	(35 h)
HI-6 [1,10]	F295L/Y337A	-	-	-	<25	(60 h)
HI-6 [10-40]	F297I/Y337A	-	-	-	<25	(60 h)
HI-6 [0.2-2]	Y337A/F338A	0.00014±0.00001	0.076±0.051	1.8±1.2	50	(40 h)
HI-6 [1-10]	BChE w.t.	-	-	-	<25	(50 h)
2-PAM [0.3-5]	AChE w.t.	-	-	-	<15	(40 h)
2-PAM [0.5-5]	Y337A	0.0007±0.0000	0.38±0.08	1.8±0.4	60	(35 h)
2-PAM [5,30]	F295L/Y337A	-	-	-	<25	(60 h)
2-PAM [20-60]	F297I/Y337A	-	-	-	<25	(60 h)
2-PAM [0.3-5]	Y337A/F338A	-	-	-	<15	(60 h)
2-PAM [3-30]	BChE w.t.	0.0079±0.0011	13±4	0.62±0.22	80	(20 h)

[a] Constants (± standard errors) are calculated (Eqns. (1)-(3)) from k_{obs} constants (4-8 values) obtained in 1 or 2 experiments.

KEY RESEARCH ACCOMPLISMENTS

- Developed further an understanding of the residue determinants for oxime reactivation using HI-6 and 2-PAM. In some cases through the use of double mutants, over 100-fold enhancements of the reactivation rate can be achieved for 2-PAM. A combination of these mutants and oximes forms a potential approach to scavenging organophosphates after exposure.
- Developed a fluorescence detection system for organophosphate exposure that not only will detect organophosphate exposure, but in some cases will distinguish between organophosphates.
- Analyzed solution conformation of AChE by steady-state fluorescence and decay of fluorescence anisotropy. The solution conformation shows significant departures from the crystal structure and forms the basis for suggesting that open and closed gorge conformations are vital to AChE catalysis and inhibition.
- Quantitated organophosphate inhibition, aging and reactivation by isolating the conjugated and free active center tryptic peptides and analyzing them by MALDI mass spectrometry. Demonstrated that this method has sufficient sensitivity to measure conjugated AChE in a single mouse brain.
- As an adjunct, we have developed or are developing four transgenic lines, in which alternative exons 5, 6 and 5 plus 6 and a regulatory region in an upstream enhancer element have been removed from the AChE gene. Breeding will enable to modify expression in a tissue and developmentally specific fashion. Other related studies that have been contributory include the crystallographic studies of structure conducted with Pascale Marchot and Yves Bourne in Marseille and studies employing click chemistry with Warren Lewis, M.G. Finn and Barry Sharpless at the Scripps Research Institution in La Jolla (See references).

REPORTABLE OUTCOMES

Publications marked by an asterisk in the reference section are reported outcomes in the open literature.

CONCLUSIONS

These accomplishments warrant future continuation along similar lines of endeavor and the following studies are projected for the next project period. Projected future studies are detailed below.

PROJECTED FUTURE STUDIES

1. The studies on active center gorge dynamics will explore other residues outside of the omega loop to ascertain the level of their fluctuations. A loop encompassing Gly 342, Gly 345, and Asp 349 will be the first candidates. Other loops connected to the active center phenylalanines that outline the acyl pocket will be an obvious second choice. Also studies are underway with double mutants where

the active site serine (Ser 203) has been removed in addition to the substitution of the cysteine at omega loop positions elsewhere for labeling. These studies should enable us to examine the influence of reversible organophosphate binding on the conformation. We hope this approach will yield a more comprehensive analysis of fluctuations in the enzyme. Where necessary, our studies will be buttressed with crystallographic studies through our long-standing collaboration with Drs. Pascale Marchot and Yves Bourne in Marseille (see enclosed references).

2. Studies with the organophosphate conjugates will be developed to develop a chip or array-based assay that relies on the fluorescence emission differences to detect organophosphate exposure.

3. Studies on inactivation and reactivation have yielded a satisfactory mutant to be tested in combination with an oxime for its potential as a scavenger. Such studies are being considered with Dr. B.P. Doctor at Walter Reed Army Institute of Research. Other mutagenesis and reactivation studies are being contemplated for analysis of fine topography of the active center and to examine other reactivation and scavenging agents. In this connection, we would activate plans with Donald Maxwell at Edgewood to also develop means for understanding intimate mechanisms of reactivity and potential scavenging and antidotal therapy for agents classified as emerging threats.

4. The transgenic animal studies are a long-term endeavor that will require seeking future support in the form of an additional proposal. Conditional knockouts of the various alternatively spliced exons and regulatory regions would be extremely valuable additions to the cholinesterase field.

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